Asialotransferrin—An Alternative to Carbohydrate-deficient Transferrin?

To the Editor:

Asialotransferrin, monosialotransferrin, and disialotransferrin are collectively referred to as carbohydrate-deficient transferrin (CDT) *(1, 2)*. Over the last several years, the definition of CDT has become increasingly vague (which transferrin isoforms are CDT isoforms and which are not). Thus, different transferrin isoforms are analyzed with various recoveries as CDT *(2)*. The lack of standardization of CDT analysis complicates the preanalysis, analysis, and interpretation of CDT. To overcome this problem, consistent use of the CDT definition introduced by Stibler *(1)*, a redefinition of CDT, or replacement by a clearly defined analyte is needed *(2)*.

In our laboratory, using isoelectric focusing for transferrin isoform analysis *(3–5)*, we observed a high prevalence of bands for asialotransferrin and monosialotransferrin, plus an increased fraction of disialotransferrin, in serum samples with increased CDT. In contrast, samples from healthy persons (with typical alcohol intake and CDT values within the reference interval) usually did not show asialo- and monosialotransferrin bands. Similar observations have been reported by others *(1, 2)*. This prompted the suggestion to replace the analyte group CDT by asialotransferrin as a clearly defined analyte *(6)*. However, there has not been a sufficiently sensitive and quantitative analytical method available to investigate the value of asialotransferrin as a marker of chronic alcohol abuse.

Recently, Legros et al. *(7)* showed that asialotransferrin (measured by capillary electrophoresis) had the best test performance for differentiating between teetotalers and alcoholics when compared with CDT (%CDT TIA assay), disialotransferrin, and asialo- + disialotransferrin (capillary electrophoresis). In their study, asialotransferrin showed the greatest area under the ROC curve. The authors pointed out that the high asialotransferrin test performance could result from the extreme differences between groups selected (teetotaler vs alcoholics). The significance of this study has been appreciated and discussed by Whitfield *(8)*.

In another study *(9)*, the same group examined moderate and excessive alcohol consumption. This report clearly showed the superior diagnostic efficiency of asialotransferrin compared with CDT, even in populations that are "closer to real life". These studies *(7, 9)* are the first experimental confirmation of the case for monitoring asialotransferrin *(6)*. Arguments for further assessment of asialotransferrin as an alternative to CDT are presented here.

The first argument is that a clearly defined analyte simplifies the validation of preanalysis (e.g., blood sampling, preparation, and shipment), analysis, and interpretation compared with an analyte group (with individual definitions of this group). Replacing the analyte group CDT by the clearly defined analyte asialotransferrin has the potential to overcome some of the basic problems of CDT analysis, e.g., consistent definition of the analyte, standardization of the analysis, and development of a direct CDT assay.

The structure of asialotransferrin can be heterogeneous, with incomplete N-glycans (lack of all terminal sialic acid molecules but presence of remnant carbohydrate chains) or with a complete lack of the N-glycans. Legros and coworkers *(7, 9)* did not examine the carbohydrate structure of the transferrin isoforms behind the different capillary electrophoresis signals. Thus, it remains unclear whether the signal assigned to asialotransferrin *(7, 9)* represents asialotransferrin with only incomplete (sialic acid-deficient) N-glycans or asialotransferrin completely lacking N-glycans. This important point needs further investigation. However, there is evidence (obtained by two independent analytical methods) of the complete lack of both N-glycans in at least some of the asialotransferrin molecules that are present in serum after chronic alcohol abuse *(2, 10, 11)*. In this case, the analyte asialotransferrin is clearly defined and does not allow individual interpretation.

The second argument is that analysis can be optimized for asialotransferrin. This will undoubtedly simplify the validation of basic analytical criteria such as analytical specificity and sensitivity (which must be higher compared with CDT analysis), detection limit, recovery, and intra- and interassay CVs.

The third argument is that asialotransferrin is not present, or is present in only trace amounts (<0.5% of total transferrin) *(2)*, under physiologic conditions.

The fourth argument is that asialotransferrin represents the transferrin isoform with the most distinct increase after chronic alcohol abuse. Serum asialotransferrin increases by 219–250%, monosialotransferrin by only 28–58%, and disialotransferrin by 148–225% *(12)*.

The fifth argument has multiple parts: (*a*) Asialotransferrin (isoelectric point ~5.9) differs from tetrasialotransferrin (isoelectric point ~5.4) by 0.5 pH units. This is of major importance because tetrasialotransferrin is the quantitatively most important (non-CDT) isoform (64–80% of total transferrin). (*b*) Disialotransferrin as the most important CDT isoform differs from tetrasialotransferrin by only ~0.2 pH units and trisialotransferrin by only ~ 0.1 pH units. As a result, coanalysis of small amounts of this non-CDT isoform can cause overestimation of CDT. (*c*) Following from (*a*) and (*b*), the analysis of asialotransferrin should be more robust against pH variations compared with CDT analysis, where slight pH differences can cause coelution or cofocusing of CDT and non-CDT isoforms *(3–5)*. (*d*) Monosialotransferrin (isoelectric point ~5.8), which is anodically next to asialotransferrin, is usually absent *(13)* or is present in only very small amounts [<0.9% of total transferrin *(12)*]. Under conditions of complete transferrin-Fe^{3+} saturation, there is no transferrin isoform cathodically next to asialotransferrin. Thus, significant cofocusing or coelution of asialotransferrin with other transferrin iso-

forms, especially non-CDT isoforms, is unlikely.

The sixth, and final, argument is that as an analyte, asialotransferrin can simplify the production of a specific antibody for a direct assay. With a complete lack of both transferrin N-glycans *(2, 10, 11)*, the structure of asialotransferrin is different from the structure(s) of the non-CDT isoforms. Thus, there is the possibility of raising a specific antibody that binds strongly to the analyte (asialotransferrin), e.g., to an epitope near or at the original point of attachment of the carbohydrate chain(s). The availability of such an antibody against asialotransferrin would make a direct assay (without microcolumn fractionation of asialotransferrin from the other transferrin isoforms, as is currently needed for CDT and non-CDT fractionation by most of the commercial CDT assays) more probable. This would be an important step toward automation of CDT (or, more precisely, asialotransferrin) analysis.

Regardless of whether CDT or asialotransferrin is used for laboratory diagnosis of chronic alcohol abuse, the diagnosis should always be made based on inclusion of a clinical questionnaire and γ-glutamyltransferase, and not on a single CDT value alone. Strategies for increasing the reliability of CDT results by use of a screening and confirmatory method have been discussed recently in this journal *(14)*.

References

1. Stibler H. Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem 1991;37:2029–37.
2. Arndt T. Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: a critical review of preanalysis, analysis, and interpretation. Clin Chem 2001;47:13–27.
3. Hackler R, Arndt T, Kleine TO, Gressner AM. Effect of separation conditions on automated isoelectric focusing of carbohydrate-deficient transferrin and other human isotransferrins using the PhastSystem. Anal Biochem 1995;230: 281–9.
4. Arndt T, Hackler R, Kleine TO, Gressner AM. Validation by isoelectric focusing of the anion-exchange isotransferrin fractionation step involved in determination of carbohydrate-deficient transferrin by the CDTect assay. Clin Chem 1998;44:27–34.
5. Hackler R, Arndt T, Helwig-Rolig A, Kropf J, Steinmetz A, Schaefer JR. Investigation by isoelectric focusing of the initial carbohydrate-deficient transferrin (CDT) and non-CDT transferrin isoform fractionation step involved in determination of CDT by the ChronAlcol.D. assay. Clin Chem 2000;46:483–92.
6. Arndt T. Carbohydrate-deficient transferrin (CDT)—should this isotransferrin group be replaced by asialo-Fe_2-transferrin and thus standardized? [Abstract]. Alcohol Alcohol 1999;34: 447.
7. Legros FJ, Nuyens V, Minet E, Emonts P, Boudjeltia KZ, Courbe A, et al. Carbohydrate-deficient transferrin isoforms measured by capillary zone electrophoresis for detection of alcohol abuse. Clin Chem 2002;48:2177–86.
8. Whitfield JB. Transferrin isoform analysis for the diagnosis and management of hazardous or dependent drinking [Editorial]. Clin Chem 2002;48:2095–6.
9. Legros FJ, Nuyens V, Baudoux M, Boudjeltia KZ, Ruelle J-L, Colicis J, et al. Use of capillary zone electrophoresis for differentiating excessive from moderate alcohol consumption. Clin Chem 2003;49:440–9.
10. Landberg E, Påhlsson P, Lundblad A, Arnetorp A, Jeppsson JO. Carbohydrate composition of serum transferrin isoforms from patients with high alcohol consumption. Biochem Biophys Res Commun 1995;267–74.
11. Peter J, Unverzagt C, Engel WD, Renauer D, Seidel C, Hösel W. Identification of carbohydrate deficient transferrin forms by MALDI-TOF mass spectrometry and lectin ELISA. Biochim Biophys Acta 1998;1380:93–101.
12. Mårtensson O, Härlin A, Brandt R, Seppä K, Sillanaukee P. Transferrin isoform distribution: gender and alcohol consumption. Alcohol Clin Exp Res 1997;21:1710–5.
13. van Eijk HG, van Noort WL, de Jong G, Koster JF. Human serum sialo transferrins in diseases. Clin Chim Acta 1987;165:141–5.
14. Arndt T, Kropf J. Alcohol abuse and carbohydrate-deficient transferrin analysis: are screening and confirmatory analysis required? Clin Chem 2002;48:2072–4.

Torsten Arndt

Bioscientia GmbH
Konrad-Adenauer-Strasse 17
D-55218 Ingelheim, Germany
Fax 49-6132-781-428
E-mail arndt@bioscientia.de

Modification of the Colorimetric Assay for Serum Unsaturated Iron-binding Capacity

To the Editor:

We reported recently that total iron-binding capacity (TIBC) values calculated from serum iron and unsaturated iron-binding capacity (UIBC) values were significantly lower than those obtained by a direct and fully automated TIBC assay *(1, 2)*. We also reported that slopes of regression lines for calculated TIBC values plotted against serum transferrin (TRF) were ~7% lower than the theoretical ratio of TIBC to TRF (TIBC/TRF = 25.1 μmol/g). We found that this could be attributed to underestimation of UIBC values by colorimetric methods. One possible reason for underestimation of UIBC values was insufficient saturation of TRF. We modified the assay conditions of a colorimetric method for UIBC measurement to improve the correspondence between TIBC values converted from TRF and those calculated from serum iron and UIBC.

Both serum iron and UIBC were determined by colorimetric methods (Wako Pure Chemical Industries) with a Hitachi Model 7070 automated analyzer. Serum TRF concentrations were determined by a nephelometric assay on a Behring Nephelometer II analyzer (Dade Behring). UIBC values were determined by four modified methods (Table 1). $UIBC_A$ was measured by the original method, in which assay conditions were set by the manufacturer. Incubation time for saturation of TRF was extended from 5 min to 10 min for the measurement of $UIBC_B$. The ratio of iron provided to saturate TRF per serum sample (iron/serum) was increased from 0.195 μmol/L to 0.26 μmol/L for the measurement of $UIBC_C$ by decreasing sample volume from 20 μL to 15 μL. Finally, both incubation and the iron/serum ratio were increased for the measurement of $UIBC_D$. TIBC values were calculated as the sum of serum iron and each UIBC value and designated Cal-$TIBC_A$, Cal-$TIBC_B$, Cal-$TIBC_C$, and Cal-$TIBC_D$, respectively. TIBC values converted from serum TRF concentrations with the theoretical TIBC/TRF ratio were designated Con-TIBC. We also calculated Cal-UIBC values as the difference between Con-TIBC and serum iron values. Correlations were assessed by principal component regression analysis. The 95% confidence interval (95% CI) for the slope of a regression line was estimated by the bootstrap method. A significance